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The pathogenicity of a newly discovered human mycoplasma (strain G37) for the genital tract of marmosets

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SUMMARY

In an attempt to demonstrate the pathogenicity of a newly discovered mycoplasma (strain G37) isolated from the human genital tract, six female marmosets (*Callithix jacchus*) were inoculated intravaginally. Four of the animals were infected as indicated by repeated recovery of the organisms on vaginal swabbing, and infection persisted for 72–149 days or more. In addition, the infected marmosets exhibited a serum antibody response detected most easily by an immunofluorescence technique, and a persistent vaginal polymorphonuclear leucocyte response not seen in two uninfected and in two uninoculated animals.

INTRODUCTION

Chlamydia trachomatis organisms cause about 50% of cases of acute non-gonococcal urethritis (NGU) (Taylor-Robinson & Thomas, 1980) and *Ureaplasma urealyticum* organisms (ureaplasmas) are responsible for the disease in some patients too, although the proportion is unknown (Taylor-Robinson & McCormack, 1980). However, it is not possible to isolate either of these micro-organisms from about a quarter of patients who suffer from NGU. Nevertheless, some of these patients respond clinically to tetracycline therapy which suggests that another tetracycline-sensitive micro-organism might infect the urethra and be responsible for NGU. During the course of a search for such an organism, a glucose-fermenting mycoplasma was isolated from the urethra of two of 13 men with NGU (Tully *et al.* 1981; Taylor-Robinson *et al.* 1981). This mycoplasma was found to be distinct serologically from all others so far known and has biological and morphological characteristics which suggest that it is pathogenic. In an attempt to evaluate its pathogenicity, we have inoculated the lower genital tract of female marmosets and the results of this investigation are described.

MATERIALS AND METHODS

Mycoplasma medium and inoculum

The mycoplasma (strain G37) had been isolated originally from the urethra of a patient with NGU. The strain was recovered in SP4 broth medium, the constituents of which have been described previously (Tully *et al.* 1979), and had

been cloned three times and passed once in SP4 medium before the inoculum was prepared for the marmosets. This was produced by inoculating three plastic tissue-culture flasks of 30 ml capacity (Nunc), each containing 8 ml of SP4 medium, with mycoplasma strain G37 and incubating them at 37 °C for 10 days, at which time a confluent layer of mycoplasmal growth was adherent to the plastic surface. Then the medium was decanted and replaced with 4 ml of phosphate-buffered saline (PBS, pH 7·2) and a few glass beads were added to the flasks which were shaken vigorously on a Whirlimix to remove organisms adherent to the plastic. The suspensions were pooled and centrifuged (M.S.E. Super Minor) at 4000 r.p.m. for 5 min. The deposit was resuspended in PBS and centrifuged as before, after which the reformed deposit was resuspended in 5 ml of PBS and the suspension stored in 1 ml aliquots at −70 °C. After rapid thawing, an aliquot was found to contain 5×10^6 viable organisms (colour-changing units: c.c.u.).

Assessment of whether the marmosets were infected vaginally by ureaplasmas was made with medium and procedures described previously (Furr, Taylor-Robinson & Hetherington, 1976).

Marmosets and inoculation

Marmosets (*Callithrix jacchus*) were bred at the Clinical Research Centre and maintained as described previously (Furr *et al.* 1976). Adult female animals, 200–300 g, were caged separately for experimental purposes. Before inoculation, the animals were bled and vaginal swabs were taken to be examined for mycoplasmas and ureaplasmas and, on several occasions, for vaginal cytology as described below. Four animals, unsedated and restrained manually, were inoculated intravaginally by introducing 0·1 ml of the mycoplasma strain G37 suspension through a cat catheter (Portex, Hythe, Kent). Two other animals were inoculated as follows: a calcium-alginate swab (Calgiswab type 1; Inolex, Illinois, U.S.A.) was inserted into the vagina of marmoset no. 204 fifty-six days after it had been inoculated with mycoplasma strain G37 and then transferred to the vagina of the recipient animal, and expressed therein.

Isolation and identification of mycoplasmas

The marmosets were restrained manually and a calcium-alginate swab (Calgiswab type 1) was inserted into the vagina. The swab was expressed in 1·8 ml of SP4 broth medium which was deemed to provide a ten-fold dilution. This was frozen at −70 °C and transported on cardice to the laboratory. Later, the samples were thawed rapidly at 37 °C and serial ten-fold dilutions (0·2 ml in 1·8 ml) were made in SP4 medium and these were incubated at 37 °C until the colour of the medium changed from pink to yellow, indicative of mycoplasmal growth. The highest dilution at which there was a colour change was considered to contain one c.c.u. and the number of organisms in a swab specimen was expressed as c.c.u./0·2 ml. Isolates were identified by the metabolism-inhibition technique using a specific rabbit antiserum (Taylor-Robinson *et al.* 1966).

Antibiotic treatment

Terramycin syrup (calcium dioxytetracyline 25 mg/ml) was administered orally to two marmosets 58 days after mycoplasmal inoculation. A 0·25 ml dose was given

twice daily for five days. Strain G37 mycoplasma organisms were inhibited *in vitro* by several tetracycline preparations at concentrations of $\leqslant 0{\cdot}001$ μg/ml.

Cytological examination

A calcium-alginate swab (Calgiswab type 1) was inserted into the vagina and then rolled on a glass slide to produce two smears. The smears were fixed in methanol, stained with Giemsa, and examined microscopically for epithelial cells and polymorphonuclear leucocytes (PMNL). The total number of PMNL in the two smears was counted and graded as follows: ±, < 50 PMNL; +, 50–300; + +, 301–1000; + + +, 1001–2000; + + + +, 2001–5000 or more.

Control animals

Two uninoculated marmosets, used for control purposes, were caged separately and swabbed for mycoplasmal isolation and cytological examination each time samples were collected from the inoculated animals.

Detection of antibody

Serum antibody titres were measured by the metabolism-inhibition (MI) technique (Taylor-Robinson *et al.* 1966) and also by an immunofluorescence (IF) procedure. In the latter, mycoplasma strain G37 antigen prepared in the same way as that for animal inoculation was applied to microscope slides in the form of spots which were allowed to dry and were then acetone fixed. Two-fold dilutions of the marmoset sera in PBS were placed on the antigen spots and incubated for 30 min in a moist chamber at 37 °C. The slides were then washed three times in PBS and a fluorescein-conjugated goat anti-human serum (Wellcome Reagents) was applied. After incubation for 30 min at 37 °C, the slides were washed as before, the PBS for the last wash containing a few drops of trypan blue, and they were then examined microscopically under ultra-violet light. The antibody titre was regarded as the highest dilution of the serum which produced + + fluorescence on a subjective scale of − to + + + +.

RESULTS

Isolation of mycoplasma strain G37

Mycoplasma strain G37 and other mycoplasmas or ureaplasmas were not isolated from the vagina of any of the marmosets before experimental inoculation of mycoplasma strain G37. However, after intravaginal inoculation of strain G37, two marmosets which had received 5×10^5 c.c.u. of the mycoplasma became infected, in addition to two marmosets which received the strain from a marmoset infected previously. As shown in Table 1, the organisms were recovered from all, or the majority, of swabs taken from these animals over periods ranging from 72 to 149 or more days. Many specimens contained at least 10^6 c.c.u. of the mycoplasma per 0·2 ml, but the number of organisms isolated varied from specimen to specimen and also from one animal to another. The detailed events before and after inoculation of one of the marmosets (no. 204) are shown in Table 2. It is of interest that the organisms were not eradicated from this marmoset, nor from marmoset no. 201, by a five-day course of Terramycin.

Table 1. *Vaginal infection in marmosets inoculated with mycoplasma strain* G37

Animal number	Inoculum (c.c.u.)	Method of inoculation	Number of swabs positive/ number taken	Maximum number organisms recovered (c.c.u./0·2 ml)	Duration of infection (days)
201	5×10^5	Catheter	16/22	10^4	72
204	5×10^5	Catheter	31/31	$\geqslant 10^6$	$\geqslant 149$
203	?*	Swab	13/15	$\geqslant 10^6$	$\geqslant 93$
206	?*	Swab	11/11	$\geqslant 10^6$	$\geqslant 93$
346	5×10^3	Catheter	0/6	—	—
524	5×10^3	Catheter	0/8	—	—

* Unknown, but swab taken from marmoset no. 204 known to be infected with at least 10^5 organisms (c.c.u.)/0·2 ml of swab specimen on the day of transfer.

A further two marmosets were inoculated with a one hundred-fold dilution of the mycoplasma strain G37 inoculum (5×10^3 c.c.u.). This failed to infect the animals since the organisms were not recovered from up to eight swab specimens taken from them over a period of one month (Table 1). In addition, strain G37 was not isolated from the vagina of two uninoculated marmosets which served as controls.

Cytological responses

Few or no PMNL were found in vaginal smears before inoculation of mycoplasma strain G37. After inoculation, none of the marmosets developed an overt vaginal discharge. However, 2–4 weeks after inoculation, increasing numbers of PMNL were seen in vaginal smears prepared from swabs taken from all of the four infected animals (Tables 2 and 3). The cytological response was intermittent and mild in two of the marmosets (nos. 201 and 206), but less intermittent and more severe (Plate 1) in the other two animals (nos. 203 and 204). The PMNL were detected for seven or more weeks in smears from three of the infected animals. Very few PMNL were seen in six specimens taken over 52 days from each of the two marmosets (nos. 346 and 524) which were inoculated with mycoplasma strain G37 but which did not become infected, or in 20 specimens taken from the two uninoculated animals over periods of 53 days and 81 days, respectively.

Antibody responses

Antibody was detected in low titre by both the MI and IF techniques in pre-inoculation sera from three of the four marmosets that became infected (Table 4). Subsequently, a four-fold serological response was seen in three of the animals by the MI technique. Furthermore, an increase in antibody titre, as measured by the IF technique, was seen also in the sera of three of the marmosets and the responses were greater than those detected by the MI technique. By the use of both methods, a slowly developing four-fold or greater antibody response was detected in all four of the infected marmosets, the magnitude being greatest in those animals (nos. 203 and 204) with the most severe PMNL response.

Table 2. *Recovery of mycoplasma strain G37 from marmoset no. 204 and its serological and cytological response to infection*

		Pre-inoculation			Post-inoculation												
Days		12	9	5	0	7	14	21	28	35	42	49	56	63	68	133	149
Organisms isolated*		Nil	·	·	·	10^2	10^4	$\geq 10^6$	10^5	10^4	10^5	10^4	10^5	$\geq 10^4$	$\geq 10^3$**	NT	10^4**
Antibody titre (reciprocal)	MI	·	·	·	2	·	·	·	·	·	·	·	8	·	·	8	·
	IF	·	·	·	2	·	·	·	·	·	·	·	2	·	·	64	·
PMNL in vaginal smear†		−	−	−		−	+	±	+++	++	++	++++	++++	++	+++	NT	NT

* Number/0·2 ml of swab specimen. ** identified serologically as strain G37. † see Materials and Methods for significance of grading.
NT, = Not tested.

Terramycin given from days 58 to 63 after inoculation.

Table 3. *Polymorphonuclear leucocyte response of marmosets inoculated with mycoplasma strain G37*

Animal number	Response starting on indicated day after inoculation	Number of vaginal smears positive*/number tested	Maximum severity of response†	Duration of response (weeks)
201	15	3/16	++	3
204	14	12/16	++++	9 or >
203	31	10/14	++++	7 or >
206	31	9/15	++	7 or >
346	−	0/6	±	−
524	−	0/6	±	−

* $\geq$ 50 PMNL/smear. † See Materials and Methods for significance of grading.

Table 4. *Serum antibody responses of marmosets inoculated with mycoplasma strain G37*

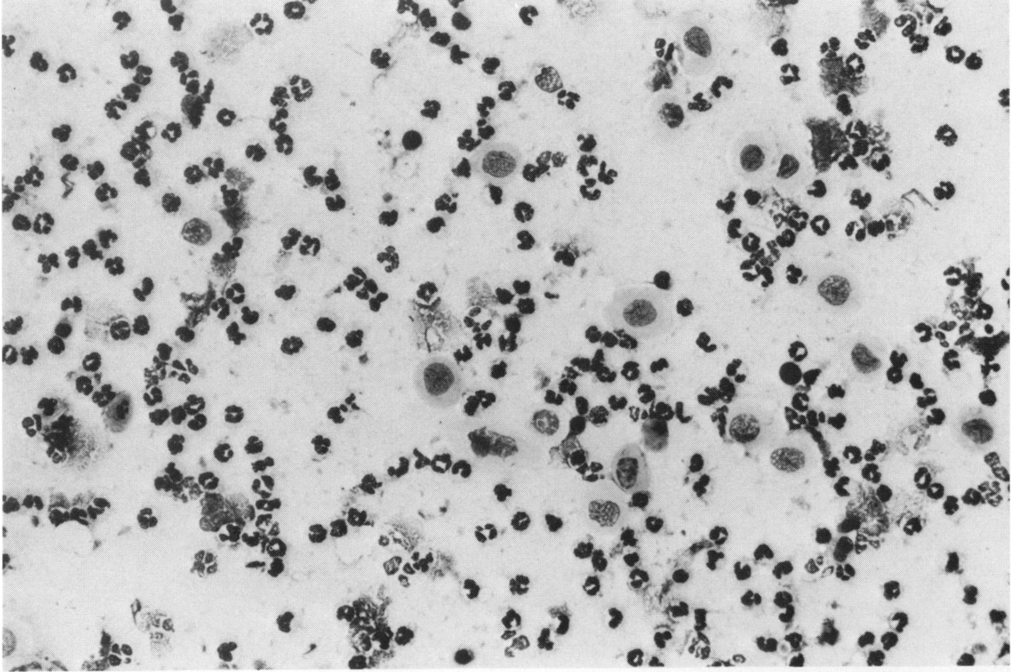
Animal number	Serological technique	Antibody titre (reciprocal) on indicated days after inoculation			
		0	55	70	133
201	MI	4	8	NS	4
	IF	2	2	NS	16
204	MI	2	8	NS	8
	IF	2	2	NS	64
203	MI	4	NS	16	NS
	IF	2	NS	128	NS
206	MI	< 2	NS	4	NS
	IF	< 2	NS	< 2	NS

NS, No serum.

DISCUSSION

We have used female marmosets previously to study genital-tract infections by ureaplasmas (Furr *et al.* 1976; Furr, Hetherington & Taylor-Robinson, 1978) and chlamydiae isolated from human subjects (Johnson *et al.* 1981). Since the animals were susceptible to these micro-organisms, we considered that it was logical to use them to study the newly discovered mycoplasma. However, as this mycoplasma was isolated from the human male genital tract, it would be desirable to inoculate male animals. The reason we have confined ourselves to experiments on female marmosets is the major difficulty, not so much of inoculation but of obtaining repeated samples from the male urethra. Clearly, it is desirable to study larger sub-human primates if this is to be achieved.

We had an insufficient number of marmosets to determine accurately the minimum infectious mycoplasma dose, but more than 5×10^3 c.c.u. were required because this number failed to infect. The donor animal used for transmitting infection by means of a swab was heavily infected, although initiation of a vaginal infection may have been assisted by adaptation of the organisms to the marmoset vagina. Susceptibility to infection may be influenced also by the hormonal status of the vaginal epithelium which we did not assess, and by pre-existing antibody which we did not find or found only in very low titre. It is of interest that minocycline therapy eliminated ureaplasmas from marmosets (Furr, Hetherington & Taylor-Robinson, 1979) but a five-day course of Terramycin failed to eradicate the strain G37 mycoplasma organisms from the vagina although they were sensitive to several tetracyclines *in vitro*. Nevertheless, infection in one of the treated marmosets was eventually self-limited. Here, as in all the animals, the infection had been long lasting. Indeed, there is no doubt that mycoplasma strain G37 produces a persistent infection in the lower genital tract of the marmoset accompanied by antibody and inflammatory cell responses. The appearance of PMNL was associated with the infection because they were rarely present, and in small numbers only, in vaginal smears before inoculation, as was the case for smears from uninfected or uninoculated animals, and they developed in large numbers in the infected marmosets, particularly in two of them. Thus, apart from the



biological features of mycoplasma strain G37, particularly those pertaining to adherence, and its morphological appearance, there is now further evidence that it has pathogenic potential. Of importance for future studies was the finding that antibody, despite its seemingly slow development in the marmosets, was detected most effectively by an immunofluorescence technique. It should be possible now to use this method to seek antibody responses to mycoplasma strain G37 in patients with NGU and other genital-tract diseases in an attempt to establish whether it has an aetiological role.

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REFERENCES

FURR, P. M., HETHERINGTON, C. M. & TAYLOR-ROBINSON, D. (1978). Studies of the specificity of ureaplasmas for marmosets. *Journal of Medical Microbiology* **11**, 537–540.

FURR, P. M., HETHERINGTON, C. M. & TAYLOR-ROBINSON, D. (1979). Ureaplasmas in the marmoset (*Callithix jacchus*): transmission and elimination. *Journal of Medical Primatology* **8**, 321–326.

FURR, P. M., TAYLOR-ROBINSON, D. & HETHERINGTON, C. M. (1976). The occurrence of ureaplasmas in marmosets. *Laboratory Animals* **10**, 393–398.

JOHNSON, A. P., OSBORN, M. F., THOMAS, B. J., HETHERINGTON, C. M. & TAYLOR-ROBINSON, D. (1981). Immunity to reinfection of the genital tract of marmosets with *Chlamydia trachomatis*. *British Journal of Experimental Pathology* **62**, 606–613.

TAYLOR-ROBINSON, D. & MCCORMACK, W. M. (1980). The genital mycoplasmas. *New England Journal of Medicine* **302**, 1003–1010.

TAYLOR-ROBINSON, D., PURCELL, R. H., WONG, D. C. & CHANOCK, R. M. (1966). A colour test for the measurement of antibody to certain mycoplasma species based upon the inhibition of acid production. *Journal of Hygiene* **64**, 91–104.

TAYLOR-ROBINSON, D. & THOMAS, B. J. (1980). The role of *Chlamydia trachomatis* in genital-tract and associated diseases. *Journal of Clinical Pathology* **33**, 205–233.

TAYLOR-ROBINSON, D., TULLY, J. G., FURR, P. M., COLE, R. M., ROSE, D. L. & HANNA, N. F. (1981). Urogenital mycoplasma infections of man: A review with observations on a recently discovered mycoplasma. *Israel Journal of Medical Sciences* **17**, 524–530.

TULLY, J. G., ROSE, D. L., WHITCOMB, R. F. & WENZEL, R. P. (1979). Enhanced isolation of *Mycoplasma pneumoniae* from throat washings with a newly modified culture medium. *Journal of Infectious Diseases* **139**, 478–482.

TULLY, J. G., TAYLOR-ROBINSON, D., COLE, R. M. & ROSE, D. L. (1981). A newly discovered mycoplasma in the human urogenital tract. *Lancet* **1**, 1288–1291.

EXPLANATION OF PLATE

Polymorphonuclear leucocytes in part of a vaginal smear of marmoset no. 203 taken 39 days after inoculation (mag. ×400); total smear contained more than 2000 leucocytes.